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Formulations for Natural and Peptide Nucleic Acids Based on Cationic Polymeric Submicron Particles

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ABSTRACT

This article describes the production and characterization of cationic submicron particles constituted with Eudragit RS 100, plus different cationic surfactants, such as dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$) and diisobutylphenoxyethyl-dimethylbenzyl ammonium chloride (DEBDA), as a transport and delivery system for DNA/DNA and DNA/peptide nucleic acid (PNA) hybrids and PNA-DNA chimeras. Submicron particles could offer advantages over other delivery systems because they maintain unaltered physicochemical properties for long time periods, allowing long-term storage, and are suitable for industrial production. Submicron particles were characterized in terms of size, size distribution, morphology, and zeta potential. Moreover, the in vitro activity and ability of submicron particles to complex different types of nucleic acids were described. Finally, the ability of submicron particles to deliver functional genes to cells cultured in vitro was determined by a luciferase activity assay, demonstrating that submicron particles possess superior transfection efficiency with respect to commercially available, liposome-based transfection kits.

KEYWORDS: peptide nucleic acids, delivery, submicron particles

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INTRODUCTION

Peptide nucleic acids (PNAs) have recently gained interest as biological response modifiers in pharmacogenetics.[1] PNAs are based on a pseudopeptide (polyamide) backbone constituted of N-(2-aminoethyl)glycine units[1-5] and exhibit (1) the ability to hybridize with high affinity to complementary sequences of single-stranded nucleic acids, such as RNA and DNA[1,5] and (2) the resistance to DNAses and proteinases.[4,5] Because of these properties, PNAs are interesting molecules for use in antisense gene therapy. However, unlike natural or chemically modified nucleic acids, PNAs display low cell membrane permeability[4,6] resulting in negligible intracellular concentrations. In addition, some PNAs are characterized by low water solubility and, therefore, poor bioavailability.[4] Moreover, since PNAs are neutral compounds, they cannot be delivered by conventional cationic formulations such as liposomes (as well as lipofectin, lipofectamine, etc) or microspheres.[4]

To solve these drawbacks, covalently bonded DNA/PNA hybrids (see **Figure 1**) were designed to improve the poor cellular uptake and solubility of PNAs[4] possibly exhibiting biological properties similar to those of natural DNA such as the ability to act as substrate for cellular enzymes (for instance DNA polymerases). In this respect, PNA-DNA chimeras[7,8] were found to generate PNA-DNA/mRNA hybrids recognized with high efficiency by RNase H.[4,9]

For delivery of PNAs, a recent study from our laboratory[7] demonstrated that cationic liposomes can be formulated for complexation of PNA/DNA hybrids. This could be an efficient strategy for the delivery of specially designed double-stranded molecules in which the DNA stretch is smaller than the complementary PNA segment, allowing exposure of the antisense por-

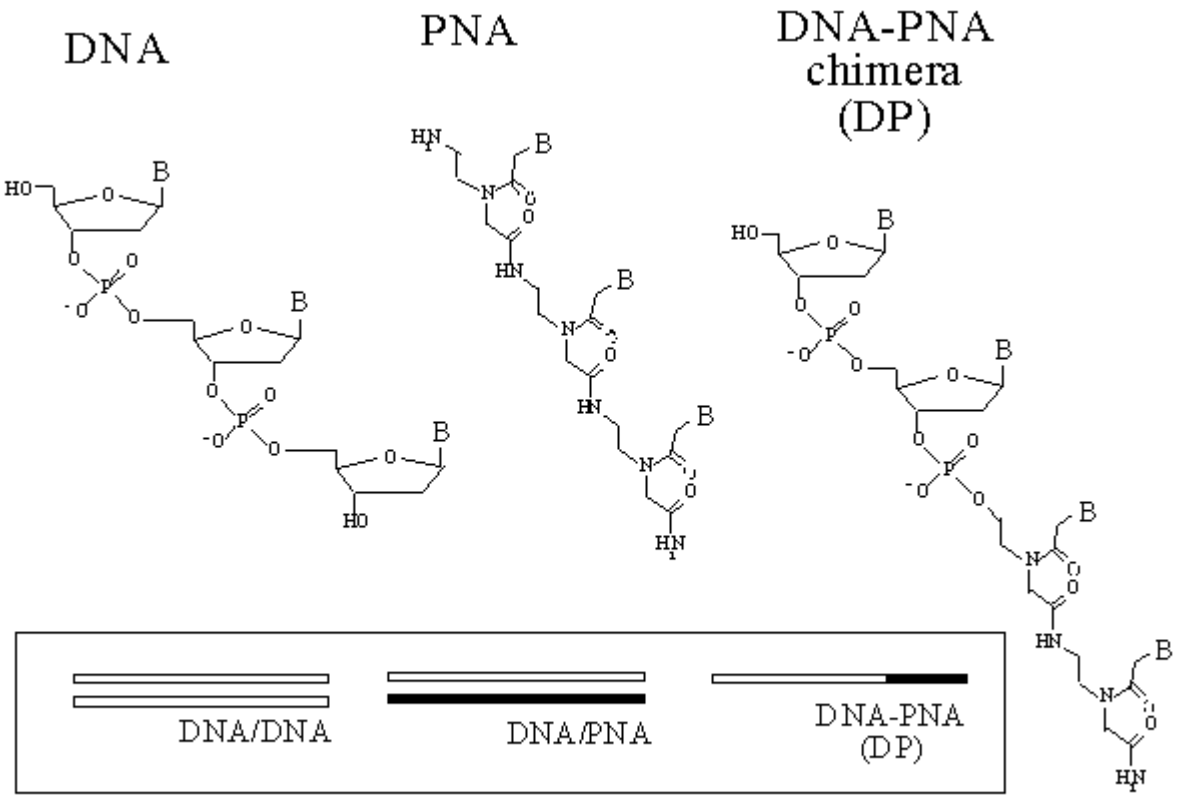

Figure 1. General chemical structure (A) and sequence (B) of the nucleic acids (DNA/DNA duplex, DNA/PNA hybrids, and DNA-PNA chimera) used in the present study.

tion of PNA. Inside the cell, this hybrid is expected to be unstable, as recently reported,[4-6] allowing the delivery of full-length antisense PNA. Still, this approach may not be suitable for highly efficient antisense gene therapy since PNA/mRNA hybrids are not efficient substrate for RNase H.[4] On the other hand, PNA-DNA chimeras are biomolecules composed of a segment of DNA covalently attached to a PNA segment,[8,9] effectively mimicking biological functions of DNA molecules.[10,11]

The major aim of this study was to determine whether cationic submicron particles constituted with Eudragit RS 100 plus different cationic surfactants, such as dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$)[12-14] can complex DNA/PNA hybrids and PNA-DNA chimeras. Submicron particles could offer a number of advantages over other delivery systems because they maintain unaltered physico-chemical properties for long time periods, allowing long-term storage, and are suitable for industrial production. In addition, depending on their composition, they could be administered via different routes (oral, intramuscular, or subcutaneous).

Finally, colloidal drug carriers, such as submicron particles, are efficiently taken up by phagocytic cells such as macrophages and tumor cells[15,16] and tend to accumulate in lysosomes. Therefore, submicron particles appear to be useful as colloidal drug carriers for PNA/DNA hybrids and PNA-DNA chimeras, for increasing their intracellular uptake.

MATERIALS AND METHODS

Materials

The polymer used for nanoparticle preparation was the acrylic copolymer Eudragit RS 100 (Rohm Pharma GmbH, Darmstadt, Germany). RS 100 is a copolymer of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups. The ammonium groups are present as salts and make the polymers permeable. The average molecular weight of the polymer is approximately 150 000 d. Eudragit RS 100 is a pH-independent polymer characterized by low permeability, useful for preparing sustained-

release formulations. The solubility is as follows: 1 g of RS 100 dissolves in 7 g aqueous methanol, ethanol, and isopropyl alcohol (containing ~3% water), as well as in acetone, ethyl acetate, and methylene chloride to give clear to cloudy solutions.

The cationic surfactants dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$) and diisobutyl-phenoxyethyl-dimethylbenzyl ammonium chloride (DEBDA) (see **Figure 2A**), were from Fluka (Buchs, Switzerland). Defibrotide (DFT), a polydeoxyribonucleotide sodium salt extracted from mammalian organs with a molecular weight between 15 000 and 30 000 d was from Crinos Industria Farmacobiologica SpA (Como, Italy). Sequences of the synthetic oligodeoxynucleotides, the PNA and DNA-PNA chimers employed, were (uppercase, DNA stretch; lowercase, PNA stretch): 5'-CGC TGG GGA CTT TCC ACG G-3'; NH_2-Gly-ccg tgg aaa gtc ccc agc g-Ac and 5'-CGC TGG GGA CTT TCc acg c-Ac.

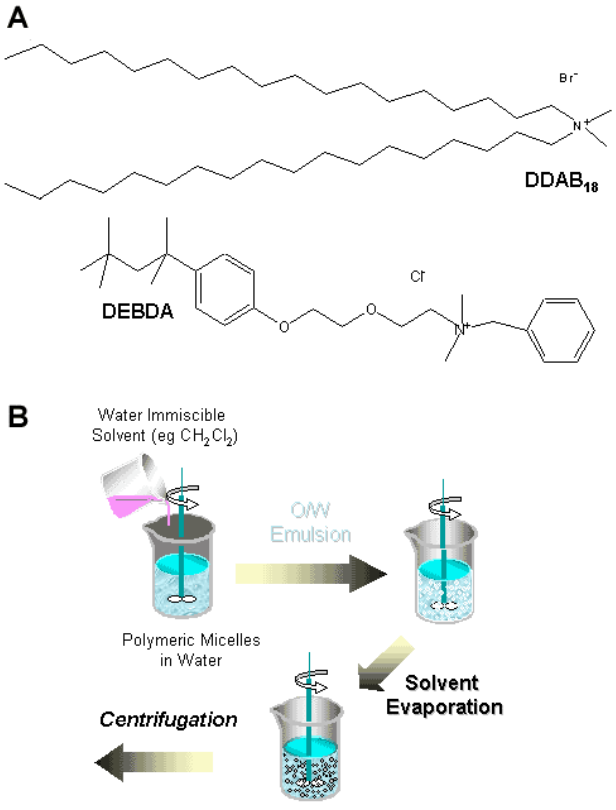

Figure 2. Chemical structure of the cationic surfactants, $DDAB_{18}$ and DEBDA, (A); and schematic representation of the solvent evaporation techniques used for the submicron particles production (B).

Methods

Production of Cationic Submicron Particles

Submicron particles were produced by a specially modified "solvent evaporation technique"[14] or by spray-drying.[13]

Solvent Evaporation Technique

Typically, 1 to 2 g of polymer plus the cationic agent were dissolved in 5 mL of CH_2Cl_2 (see **Figure 2B**) (the ratio between polymer and cationic agent was 70:30 wt/wt). The mixture was emulsified with 100 mL of an aqueous phase (oil to water ratio: 0.05, vol/vol) containing 88% hydrolyzed polyvinyl alcohol (PVA) (Airvol 205, Air Products Corp, PA) as dispersing agent. The obtained emulsion was continuously stirred at 1000 rpm. After complete evaporation of CH_2Cl_2 (usually occurring within 3-5 hours at 50°C), submicron particles were isolated by a differential centrifugation scheme, as described in **Figure 3** (see below).

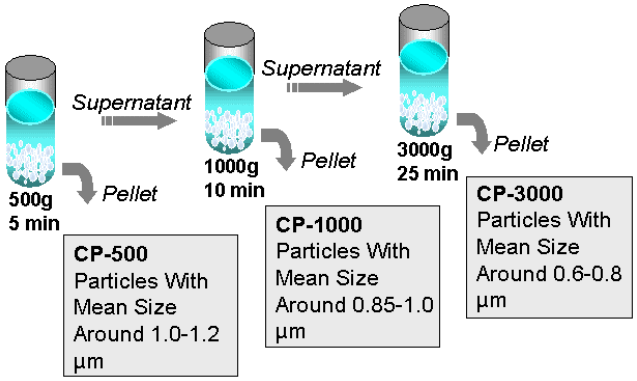

Figure 3. Schematic representation of submicron particle isolation procedure by centrifugation.

Spray-Drying

Submicron particles were produced using a Buchi Mini Spray Dryer (model 190, Buchi, Laboratoriums Technik AG, Flawil, Germany). Using a peristaltic pump (0.5 mL/min), 1.5 g of polymer solubilized in CH_2Cl_2 was fed into the instrument and sprayed with a 0.7-mm nozzle, by means of a flow of compressed air (28 m^3/h), in the drying chamber of the apparatus. A flow of heated air (50°C) aspirated by a pump (600 L/h) induced the quick evaporation of the solvent from the drops, leading to the formation of solid microparticles. After separation from the exhausted air in a cyclone, the particles settled into a bottom collector. Particles were then suspended in the water and separated in different dimensional fractions by the differential centrifugation scheme described

in **Figure 3**. Briefly, after isolation, the particles were suspended in 5 mL water and centrifuged at 500g for 5 minutes. The collected pellet was named CP-500. The supernatant was resuspended (in 5 mL water) and further centrifuged at 1000g for 10 minutes; again the pellet was collected and named CP-1000. Finally, the supernatant was centrifuged at 3000g for 25 minutes resulting in the pellet of particles named CP-3000.

In order to verify if the purification process (the washing and/or centrifugation procedures) would eventually lead to a partial release of the cationic agent from the particles (possibly causing a modification of the particle charge), a determination of the concentration of the cationic surfactant was performed both on the particles and on the collected washing phases. Both analyses (performed by high performance liquid chromatography [HPLC]) confirmed that the cationic surfactants are tightly bound to the particles, and no release of cationic surfactant was detectable. Thus, the cationic charge of the particles remains unchanged before and after the purification process. Both cationic surfactants were analyzed by HLPC. The HPLC system was Bruker apparatus (Bremer, Germany) consisting of a 3-plunger alternative pump, a variable wavelength UV detector operating at 234 nm, and a Rheodine Inc (Rheodyne LLC, Rohnert Park, California, USA) injection valve with a 10-µL loop; column. The following conditions were used: chromolith speed ROD RP-18e column (50 × 4.6 mm); mobile phase A: methanol, B: 0.1% TFA; gradient: time 0 minutes, A = 60%, B = 40%; time 2.5 min, A = 100%; flow rate: 4 mL/min; UV; injection volume 10 µL.

Optical and Electron Microscopy Analysis

Submicron particle morphology was determined by electron microscopy observations. For the electronic analysis, microparticles were metallized by gold coating (Edwards Sputter coating S150, BOC (Edwards High Vacuum International, Wilmington, MA, USA) and analyzed at 15-20 Kv by a scanning electron microscope (SEM) 360 Stereoscan (Cambridge Instruments Ltd, Cambridge, UK).

Determination of Submicron Particle Size and Zeta Potential

Nanoparticle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instruments, Malvern, England), equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements were made at 25°C at an angle of 90°. Data were interpreted using the Contin software (Malvern Instruments, Malvern, England) Each sample was analyzed in triplicate, and the reported data represent the mean values.

The electrophoretic mobility of cationic submicron particles and DNA complexes was measured at room temperature using Zetasizer 3000 photon correlation spectroscopy (PCS) (Malvern Instruments) in 1mM NaCl solution (The 1-mM NaCl solution was used for zeta measurement as it is the solution recommended by the instrument producer (Mike Kaszuba, Technical support scientist, Malvern Instruments, personal communication). Samples were injected in a glass capillary cell and analyzed under a constant voltage after focusing with a 5 mW helium neon laser. The zeta potential, in mV, was automatically calculated from the electrophoretic mobility based on the Smolukowski formula.

Association of Defibrotide to Submicron Particles

Five milligrams cationic submicron particles were suspended in 3 mL borate buffer, pH 7.4, containing different amounts of DFT. The suspension was then maintained under mixing for 1 hour using an orbital Ika stirring motor (Eurostar digital, IKA-Labortechnik, Bremen, Germany), at 125 rpm. The amount of associated DFT was calculated by evaluating the concentration of the free DFT in the borate buffer after separation of the microspheres. The determination was performed by diluting the borate buffer 1:10 vol/vol and evaluating DFT concentration by ultraviolet spectrophotometric analysis (Perkin Elmer Corp, Norwalk, CT).

Synthetic Oligonucleotides, PNA, and Production of PNA-DNA Chimeras

The synthetic oligodeoxynucleotides used in this study were purchased from Sigma (St Louis, MO). PNA monomers for production of PNA-DNA chimeras were synthesized in Prof Van Boom's laboratories[8,9] (Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, The Netherlands); DNA monomers were obtained from Perseptive Biosystems (PerSeptive Biosystems Inc. Framingham MA, USA). Methanol (HPLC grade, Fluka, Buchs, Switzerland) was stored over molecular sieves (3 Å) and used without further purification. All other solvents (DNA synthesis grade, Biosolve, Valkenswaard, The Netherlands) were used as received. Automated syntheses of the chimeras were performed on a Pharmacia Gene Assembler, using highly cross-linked polystyrene (loading 26-28 µmol/g) as the solid support on a 1-µmol scale, as reported elsewhere.[10] After

the last elongation step, the oligomers were cleaved from the solid support and deprotected by treatment with 1.5 mL methanolic ammonia, at 50°C for 16 hours. The samples were filtered and then purified by reversed phase (RP)-HPLC on a LiChrosphere 100 RP-18 end-capped column (4 × 250 mm) (Merck Pharma S.p.A., Milano, Italy) on a Jasco HPLC system (JASCO Europe s.r.l, Cremella, Lecco, Italy) as reported elsewhere.[11] Gradient elution was performed at 40°, building up gradient starting with buffer A (50mM triethylammonium acetate in water) and applying buffer B (50mM triethylammonium acetate in acetonitrile/water, 1/1, vol/vol, in 20 minutes with a flow rate of 1 mL/min.

Complexation DNA/DNA, DNA/PNA Hybrids, and DNA-PNA Chimeras to Submicron Particles

The ability of cationic submicron particles to bind DNA/DNA or DNA/PNA hybrids and DNA-PNA chimeras was determined by electrophoretic mobility shift assay.[7] The sense DNA strand of DNA–DNA and DNA–PNA was end-labeled using [γ-^{32}P]adenosine triphosphate (ATP) and T4-polynucleotide kinase. Increasing amounts of submicron particles were incubated for 30 minutes in borate buffer (0.28mM $Na_2B_4O_7$, 10mM H_3BO_3, pH 7.4) with 100 ng of cold DNA–DNA, or with 100 ng of a mixture of cold plus ^{32}P-5'-end-labeled (99 ng and 1 ng, respectively) DNA–DNA or DNA–PNA molecules. After binding, electrophoresis was performed through a polyacrylamide gel; gels were dried and exposed to X-Omat ar films (Eastman Kodak Company, New Haven, CT, USA) for autoradiographic procedure.

Luciferase Assay

One milligram of submicron particles in 100 μL of borate buffer were complexed with 20 μg of pGL3 vector containing the luciferase gene under the control of the SV40 promoter. After 30 minutes binding at room temperature, the mixture was added to 5 mL (medium) of in vitro cultured J774 cells (50% confluent). After 2 days of cell culture in complete medium (including 10% serum), J774 cells were collected by a 10-minute centrifugation at 1000 rpm and washed twice with saline. The cell pellet was resuspended in 200 μL of Cell Culture Lysis Reagent (Luciferase Assay System, Promega Italia, Milan). The cytoplasmic supernatant fraction was separated by a 10-minute centrifugation at 12 000g; proteins were quantified by the Bradford procedure, and 30 μg of protein extracts, 100 μL of Luciferase Assay Substrate (Luciferase Assay System) was added. Luciferase activity was determined with an analytical luminometer (model TD-20/20, Turner Design, Sunnyvale, CA). Triplicate luciferase assays of all the samples were performed. Results are reported as *fold induction* by determining the ratio of the analyzed firefly luciferase reporter plasmid to that produced by the pGL3-basic vector lacking promoter activity.

In Vitro Cell Culture Experiments

Effect of Submicron Particles on Cell Growth

The effects of cationic submicron particles on cell proliferation were determined on in vitro cultured human leukemic K562 cells.[7] Standard conditions for cell growth were α-medium (Gibco, Grand Island, NY), 50 mg/L streptomycin, 300 mg/L penicillin, supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) in 5% CO_2 at 90% humidity. Cell growth was determined by counting with a Coulter Counter (model ZF, Coulter Electronics Inc, Hialeah, FL). Counts of viable cells were performed after 0.1% Trypan blue exclusion test.

Particle-Cell Interaction Studies (Scanning Electron Microscope Analysis)

The analysis of particle-cell interactions was performed on mouse macrophages J774 and mammalian breast carcinoma MCF7 cell lines. To subconfluent cells (cultured in 2.5 cm petri dishes), 1 mg of submicron particles was added. After 5 minutes incubation, cells were washed in triplicate with sterile saline and then fixed with a 2% solution of glutaraldehyde. Scanning electron micrographs were taken after metallization by gold coating and analyzed at 15 to 20 Kv by a scanning electron microscope (SEM).

Particle-Cell Interaction Studies (Fluorescence Analysis)

Fluorescein-labeled DNA-PNA molecules were used. Modified sense strand DNA molecules, carrying fluorescein at 5'-end-labeled and HPLC-purified, were purchased from Amersham (Amersham-Pharmacia, Milan, Italy). Murine macrophage J774 cells were incubated for 20 hours in the presence of fluorescein-labeled DNA-PNA molecules. After this incubation period, cells were fixed and observed under an Olympus fluorescence-microscope (Olympus BX60-fluorescence, Olympus Optical Company GmbH, Hamburg, Germany).

Protective Effects of Submicron Particles on Double-Stranded DNA/PNA Hybrids

DNA/DNA and DNA/PNA target molecules were incubated without or with submicron particles for 30 minutes at room temperature, and then serum (fetal calf serum, Eurobio, France) or K562 cellular extracts were added (3 µL/reaction for serum and 1 µg/reaction for cellular extracts). Cellular extracts from human K562 cells were prepared as described elsewhere.[11] Protein concentration of FCS was 30 g/L, according to the manufacturer. After overnight incubation, the reactions were phenol extracted, ethanol-precipitated, and electrophoresed, and autoradiography was performed.

RESULTS AND DISCUSSION

Nucleic Acids

Figure 1 depicts the chemical structure of DNA, DNA/PNA hybrids and PNA-DNA chimeras used in the present study. Our hypothesis is that PNA can be delivered to target cells in hybrid form with DNA, either intermolecularly (as in the case for DNA/PNA hybrids) or intramolecularly (as in the case for DNA-PNA chimera). In the present study, we mainly focused on PNA-based molecules of possible interest for nonviral antisense gene therapy, where the target mRNA should be efficiently recognized by the therapeutic molecules. Since PNA/RNA hybrids are not substrate for RNase H (ie, the target RNA sequence is not degraded by the enzyme), antisense PNAs, carried by DNA stretches, could be useful in all applications where target mRNA[7] should not be degraded. On the contrary, PNA-DNA antisense chimeras should be considered for efficient RNase-H-dependent degradation of PNA-DNA/RNA hybrids.[4]

Preparation of Cationic Submicron Particles

Cationic polyacrylic submicron particles were prepared using different excipient compositions and manufacturing procedures with the aim to study the effect of the experimental parameters on the resulting submicron particles.

Typically, a blend between a permeable polymer and a cationic surfactant was used, and as manufacturing procedures, (1) solvent evaporation or (2) spray-drying techniques were employed. Eudragit RS is a copolymer of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups. The ammonium groups are present as salts and make the polymer permeable. Two different cationic surfactants were employed, namely, $DDAB_{18}$ or DEBDA (**Figure 2A**).

The experimental section reports the settings used for solvent evaporation[14] and spray-drying[13] techniques. Optimal settings for both methods[13,14] were chosen, following previous experiments that allowed the production of submicron particles with good characteristics in terms of shape, polydispersity, and absence of aggregation.

Special attention was paid to the isolation of submicron particles. However, submicron dimensions did not allow easy and fast application of common filtration strategies. A differential centrifugation protocol was established (see **Figure 3**) for isolating the particles, obtaining 3 subpopulations of different sizes (named CP-500, CP-1000, and CP-3000, respectively) reflecting the spinning force of the centrifuge (in g). Residues derived from the preparation procedure were washed out, with special regard to PVA used for the solvent evaporation procedure. **Table 1** reports the total and relative recovery efficiencies for the different nanoparticle batches and the preparation time required for both preparation procedures. In addition, **Table 1** provides general characteristics of submicron particles, such as mean diameter, recovery, and zeta potential of the different nanoparticle subpopulations. The largest proportion (by weight) of submicron particles was isolated at 500g, while submicron particles isolated at 3000g represent the smaller subpopulation, not exceeding 15% of the total particle amount. The percentage of particle recovery by weight, with respect to the total amount of polymer used, was satisfactory for the solvent evaporation technique, while the spray-drying yields were lower, not exceeding 55%.

Figure 4 also summarizes the size distribution of the cationic submicron particles. The cumulative undersize frequency distributions for $DDAB_{18}$- and DEBDA-based submicron particles produced by solvent evaporation are provided in **Figures 4A** and **B**. All submicron particles are characterized by a relatively narrow, normal size distribution with a mean diameter (by number) of 1.0, 0.87, and 0.66 µm (for $DDAB_{18}$) and of 1.08, 0.94, and 0.88 µm (for DEBDA) for CP-500, CP-1000, and CP-3000, respectively. All submicron particles carried a net positive charge, as demonstrated by the zeta potential measurements being between +14 and +54 mV (see **Figure 3**), even if DEBDA-based submicron particles generally present a lower positive charge with respect to $DDAB_{18}$ ones.

Submicron Particles Characterization

Figure 5 shows the electron photomicrographs of the produced cationic submicron particles. In particular, panels A and B depict submicron particles prepared by

Table 1. Comparative Analysis of Microparticle Characteristics as Function of the Production Approach*

Eudragit RS/$DDAB_{18}$		
Characteristics	**Solvent Evaporation**	**Spray-Drying**
Preparation time	5 hours	10 minutes
Total recovery† (%)	66.2	53.0
Relative recovery‡ (%) by dimensional classes	64.5 (500g) 24.1 (1000g) 11.4 (3000g)	88.0 (500g) 7.8 (100g) 4.2 (3000g)
Mean dimension (μm) relative to 1000g	0.87	1.15
Zeta potential (mV) by dimensional classes	+32.1 (500g) +28.7 (1000g) +27.9 (3000g)	+54 (500g) +41 (1000g) +35 (3000g)
DFT association (%) +/- molar ratio 16:1	97.9	99.3

Eudragit RS/ DEBDA		
Characteristics	**Solvent Evaporation**	**Spray-Drying**
Preparation time	5 hours	10 minutes
Total recovery† (%)	64.0	42.5
Relative recovery‡ (%) by dimensional classes	70.6 (500g) 23.3 (1000g) 6.1 (3000g)	93.0 (500g) 5.5 (1000g) 1.5 (3000g)
Mean dimension (μm) relative to 1000g	0.94	1.22
Zeta potential (mV) by dimensional classes	+14.5 (500g) +22.8 (1000g) +23.9 (3000g)	+22.2 (500g) +21.8 (1000g) +36.5 (3000g)
DFT association (%) +/- molar ratio 16:1	75.6	72.3

*DFT indicates defibrotide. Data represent the average of 3 independent batches.
†Percentage of submicron particles recovered with respect to the total amount of polymer used.
‡Percentage of submicron particles recovered with respect to the total amount of polymer used, after dimensional classification by centrifugation (see Figure 3).

solvent evaporation and spray-drying techniques, respectively. Both samples were directly analyzed at the end of the preparation without centrifugal fractionation, as demonstrated by the large polydispersity of the samples, even if the spherical shape, the smooth surface, and the absence of aggregates were satisfactory. Conversely, panels C and D show the smallest submicron particles (those isolated at 3000g), obtained from solvent evaporation (C) and spray-drying (D) batches, respectively. Here the particles presented with a narrower size distribution, smooth surface sufficiently spherical, and only a few collapsed, irregular particles.

Binding of DFT to Cationic Submicron Particles

In a preliminary experiment, the ability of cationic submicron particles to bind nucleic acids was investigated using DFT (molecular weight 26.200 d), a single-stranded polydesoxyribonucleotide (DNA) sodium salt extracted from mammalian organs.[17] **Figure 6A** reports DFT binding to $DDAB_{18}$-based and DEBDA-based submicron particles when complexed at different positive to negative molar charge ratios. DFT was added at charge molar ratios (+/-) between 1:1 and 16:1. The association of DFT to submicron particles followed an almost linear trend. In the case of $DDAB_{18}$-based submicron particles, the DFT association was always higher with respect to the DEBDA-based particles.

After complexation of DFT to the particles, visual inspection revealed no signs of aggregation or flocculation, either immediately after the mix or after long-term storage (observations were made up to 1 month). In order to instrumentally detect possible particle aggregation phenomena after complexing with DNA compounds, we determined the size and zeta potential of the complexes. To characterize nucleic acid binding to both types of cationic submicroparticles, size and zeta potentials of the complexes were measured at different molar charge ratios (+/-). Zeta potential is important for characterizing cationic nonviral gene-delivery systems. **Figures 6B** and **C** illustrate the size and zeta potential of cationic submicron particles before and after complexation with DFT. Before complexation, submicron particles possessed a net positive zeta potential of +29.0 mV ($DDAB_{18}$) and 22.8 mV (DEBDA); when DFT was added to the submicron particles at 16:1 (+/- molar ratio), the zeta poten-

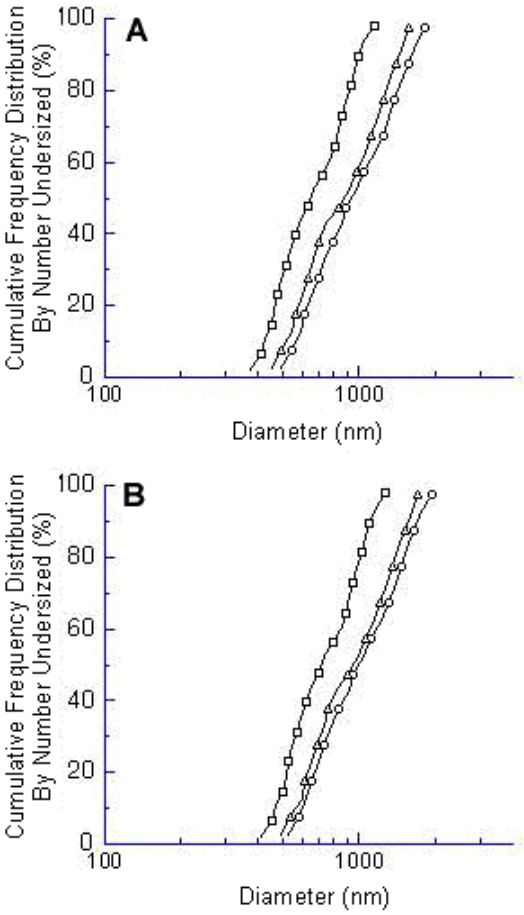

Figure 4. Cumulative frequency size distribution plot, by number (undersize), of $DDAB_{18}$-based submicron particles (panel A) and DEBDA-based submicron particles isolated by centrifugation at 500g (CP-500, circles), 1000g (CP-1000, triangles), and 3000g (CP-3000, squares). Data represent the mean of 5 independent determinations.

tial decreased to negative values to a minimum of -15.7 mV.

In Vitro Studies on Cationic Submicron Particles

An in vitro study was performed for determining the cytotoxic activity of cationic submicron particles. Human leukemic K562(S) cells were treated with different amounts of submicron particles. After 5 days of culture, cells were electronically counted. **Figure 7** reports the cytotoxic activity of both CP-1000 $DDAB_{18}$- and DEBDA-based submicron particles. The results demonstrate that $DDAB_{18}$-based submicron particles are only slightly more cytotoxic than other cationic formulations used for gene therapy, such as lipofectin, cellfectin, and lipofectamine.[7] Conversely, DEBDA-based submicron particles were more toxic displaying an IC_{50} of 125 µM. Because of their lower toxicity, $DDAB_{18}$-based submicron particles can be safely used in ex vivo experiments, and therefore were selected for further experiments of complexation with decoy or antisense molecules.

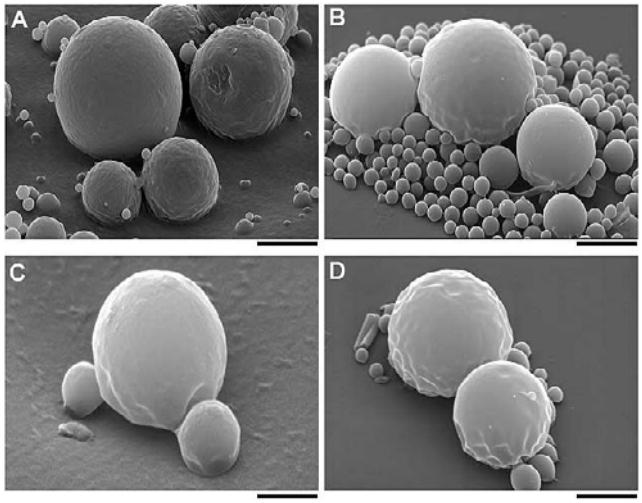

Figure 5. Scanning electron micrographs of Eudragit RS-$DDAB_{18}$ submicron particles produced by solvent evaporation (A,C) or spray-drying (B,D) techniques. Pictures were taken immediately after preparation (A,B) and after centrifugation at 1000g (C,D). Bar corresponds to 1.2 and 300 nm in panels A-B and C-D, respectively.

To assess if cationic submicron particles can efficiently interact with in vitro cultured cells, an experiment was conducted with J774 and MCF7, mouse macrophages and mammalian breast carcinoma cell lines, respectively. Cells were cultured in the presence of 100 µg/mL of CP-1000 cationic $DDAB_{18}$-based submicron particles and after 5 minutes were fixed with glutaraldehyde and observed by SEM analysis. As evident from the photomicrograph shown in **Figure 8**, cationic submicron particles rapidly and efficiently attached to the cell surface of both cell lines. In addition, the figure suggests that some particles are taken up by the cells, probably by phagocytosis. This finding supports the use of these particles as a delivery system for ex vivo experiments.

Transfection of Plasmid DNA With Cationic Submicron Particles

As a further experiment to evaluate the ability of cationic submicron particles to transport DNA molecules, a functional experiment was performed in vitro. In this experiment, 1 mg of cationic submicron particles was suspended in borate buffer and allowed to complex with the pGL3 vector containing the luciferase gene. After

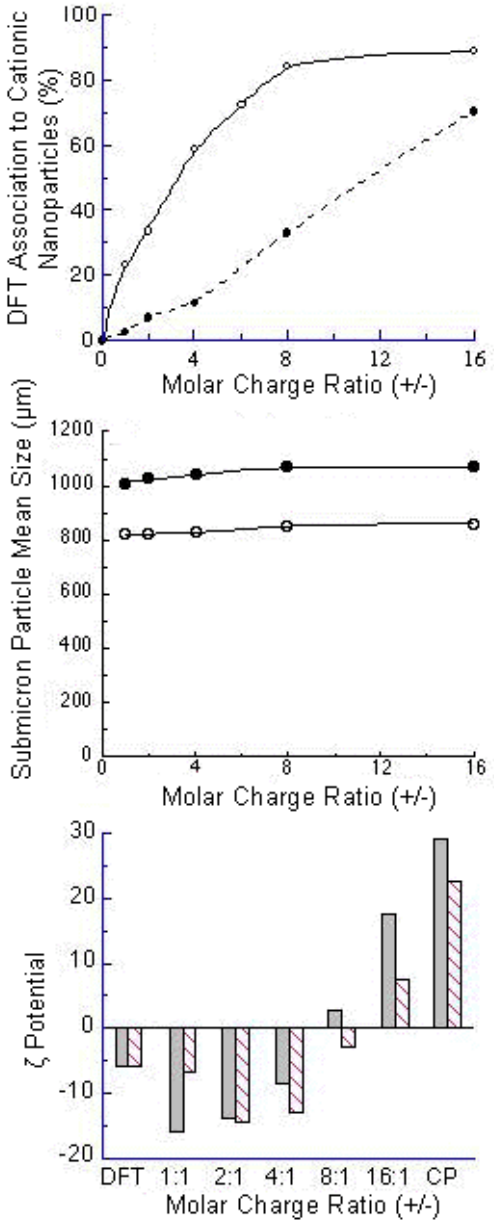

Figure 6. (Panel A) Percentage of nucleic acid (DFT) association to CP-1000 cationic $DDAB_{18}$-based submicron particles (○) and DEBDA-based submicron particles (●). Mean size (panel B) and zeta potential (panel C) of cationic submicron particles complexed to nucleic acid. CP-1000 cationic $DDAB_{18}$-based submicron particles (open bars) and DEBDA-based submicron particles (striped bars) were complexed with DNA at the indicated molar charge ratios; as a reference, the zeta potentials of free DNA (DFT) and uncomplexed submicron particles (CP) were also reported. The used submicron particles were from batch CNS#2, fraction isolated by centrifugation at 500g. Data represent the mean of 3 independent determinations.

complexation, DNA-carrying submicron particles were added to in vitro cultured J774 cells, as described in the experimental section. After 48 hours, cells were recovered, washed, lysed, and assessed for luciferase activity as described in the "Methods" section. The results reported in **Figure 9** represent the fold induction of luciferase activity of delivered plasmid with respect to the luciferase activity of free vector plasmid. In fact, submicron particles display a transfection efficiency strictly correlated to their size. CP-500 and CP-1000 induced only a slight increase of luciferase activity (2- and 8-fold increase, respectively) when compared with control untreated cells. On the contrary, the smaller CP-3000 submicron particles displayed a dramatic increase in luciferase expression up to 360-fold over the basal level. For comparison, J774 were also treated with the well-established and largely diffused lipofectin, which resulted in a 9-fold induction of luciferase gene expression.

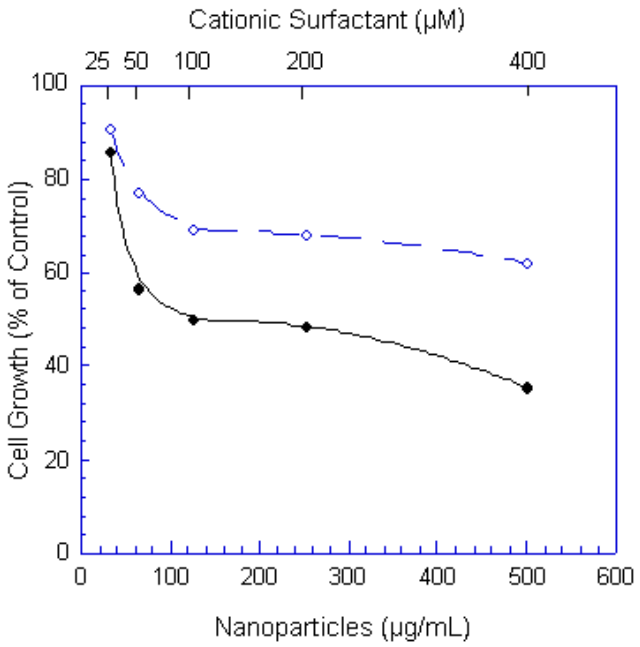

Figure 7. In vitro effect of CP-1000 cationic $DDAB_{18}$-based submicron particles (○) and DEBDA-based submicron particles (●) on cultured K562 human erythroleukemic cells. Data represent the average of 5 independent experiments and are expressed as percentage of cell number/mL compared with untreated control K562 cells after 6 days of cell culture.

Complexation of DNA/DNA, DNA/PNA Hybrids, and DNA-PNA Chimeras to Submicron Particles

Figure 10 demonstrates that $DDAB_{18}$-based submicron particles (CP-1000) are able to form stable complexes with different types of nucleic acid molecules, such as DNA/DNA (panel A), DNA/PNA hybrids (panel B), and DNA-PNA chimeras (panel C). The general ability of submicron particles to bind nucleic acids was demonstrated by gel-shift experiments. The autoradiographs of the gels display 2 major bands: (1) high molecular weight, nonmigrating bands, identified as nucleic acid bound to submicron particles, and (2) low molecular weight, migrating bands, identified as free nucleic acids. It is evident that increasing the weight ratio between submicron particles and nucleic acid leads to a progressive increase in the nonmigrating bands, indicating the complexation of the employed molecules to submicron particles. As expected, the DNA/DNA duplex is more efficiently complexed to cationic submicron particles (because of its high negative-charge density); the DNA/PNA hybrids display an intermediate complexing behavior (more strictly resembling that of DNA/DNA duplex); finally, the DNA-PNA single-stranded chimeras are the less efficiently complexed molecules. In fact even at the highest submicron particle/chimera ratio, a weight ratio, the presence of the band of free uncomplexed compounds is still evident.

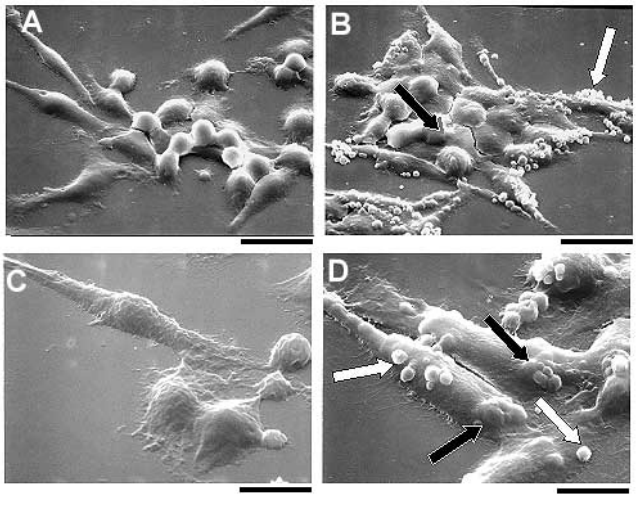

Figure 8. Scanning electron micrograph of J774 (A, B) and MCF7 (C, D) (mouse macrophages and mammalian breast carcinoma cell lines, respectively) treated with CP-1000 cationic $DDAB_{18}$-based submicron particles. Untreated control cells (A, C) and particle treated cells (B, D). White arrows indicate submicron particles attached to the cell surface, Black arrows indicate internalized (engulfed) submicron particles. Bar corresponds to 26.1, 16.7, 7.7, and 6.1 mm in panels A, B, C, and D, respectively.

In Vitro Delivery of DNA/PNA Hybrids by Cationic Submicron Particles

The experiment shown in **Figure 11** was conducted to demonstrate that DNA/PNA hybrids can be delivered to target J774 cells. In this experiment, fluorescein-labeled DNA/PNA hybrids were complexed to $DDAB_{18}$-based submicron particles (CP-3000). After 30 minutes binding at room temperature, the mixture was added to J774 cells, as described in the experimental section. After 3 hours, J774 cells were recovered, washed, and analyzed. The results support the uptake of PNA/DNA hybrids by submicron particles.

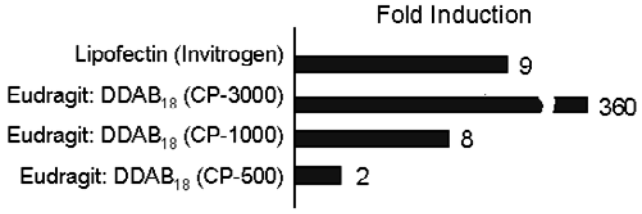

Figure 9. In vitro transfection ability of cationic $DDAB_{18}$-based submicron particles. J774 cells were treated with the same amount of pGL3 vector, containing the luciferase gene under the control of the SV40 promoter complexed to the indicated nonviral gene delivery systems (the ratio between plasmid and delivery system being 2:100, wt/wt). Lipofectin (Invitrogen) was used as control representing a well-established and largely diffused liposome-based transfection agent. The results reported represent the fold induction of luciferase activity of delivered plasmid with respect to the luciferase activity of free vector plasmid. Data represent the average of 3 independent experiments.

Protective Effects of Submicron Particles on Double-Stranded DNA/PNA Hybrids

This section addresses the question of whether cationic submicron particles protect natural or peptide-based nucleic acids from enzymatic degradation. The experiment described in **Figure 12** was performed using ^{32}P-labeled DNA or DNA/PNA hybrids. When DNA and DNA/PNA hybrids are incubated in serum, a fast, time- and concentration-dependent decrease of radioactivity is observed, caused by degradation of the DNA stretch. When the DNA or DNA/PNA hybrids are complexed with increasing amounts of $DDAB_{18}$-based cationic submicron particles before incubation with serum, they are protected from this degrading activity.

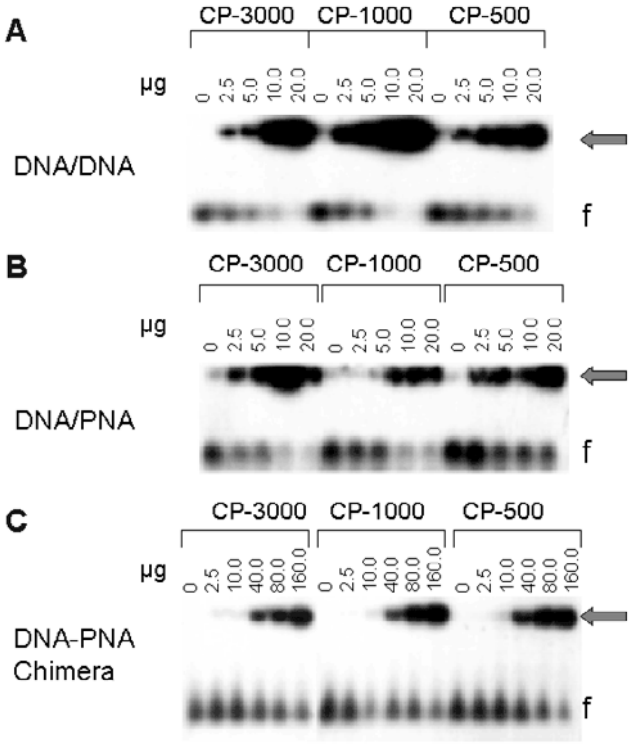

Figure 10. Comparative analysis of the ability of cationic $DDAB_{18}$-based submicron particles to complex different nucleic acid molecules: DNA duplex, DNA/PNA hybrids, and DNA-PNA chimera. The indicated amounts of CP-500, CP-1000, and CP-3000 submicron particles were incubated in the presence of the radio-labeled nucleic acids; the formed complexes were electrophoresed through an agarose gel and processed for autoradiography. Arrows indicate bound material complexed to cationic $DDAB_{18}$-based submicron particles; and f, free target molecules.

CONCLUSION

In spite of a number of possible advantages over commonly used nucleic acids, PNA present 2 major drawbacks: (1) they are not taken up by the cells,[3-6] and (2) being neutral molecules, they are not suitable for efficient delivery with nonviral cationic formulations (liposomes, dendrimers, polymers).[4] The goal of the present study was to determine whether cationic submicron particles efficiently bind PNA/DNA hybrids and PNA-DNA chimeras. PNA/DNA hybrids could serve as a prodrug if the PNA length is greater than that of the complementary DNA strand, exposing the antisense PNA stretch. On the other hand, PNA-DNA chimeras[4,7] represent an excellent candidate for antisense gene therapy,[9-11] since they are resistant to exonucleases[4]; in addition, PNA-DNA/mRNA hybrids are efficiently recognized by RNase H.[4,7]

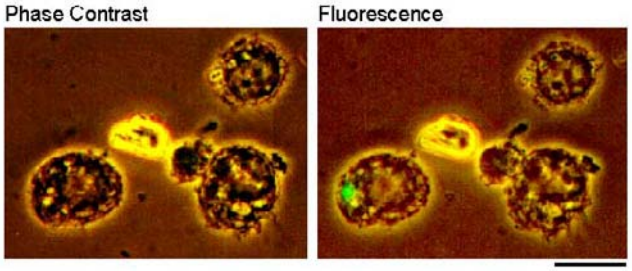

Figure 11. Microphotograph showing inclusion of a fluorescein-labeled DNA/PNA hybrid to J774 cells (confluent at a level of 50%). Bar represents 10 µm. The cells of control experiments, such as cells treated with (1) free fluorescein-labeled DNA/PNA hybrid, (2) submicron particles without the DNA/PNA hybrid, and (3) completely untreated cells, did not show any appreciable fluorescence (data not shown).

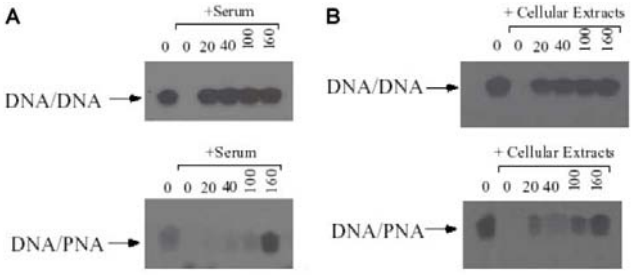

Figure 12. Protective effects of cationic $DDAB_{18}$-based submicron particles on ^{32}P-labeled DNA and DNA/PNA target molecules. Target molecules were incubated without (o) or with the indicated concentrations (20-160 µg/reaction) of $DDAB_{18}$-based submicron particles for 30 minutes at room temperature and then serum (foetal calf serum) or cellular extracts (from K562 cells) were added (3 µL/reaction). After overnight incubation, the reactions were phenol extracted, ethanol precipitated, and electrophoresed, and autoradiography was performed. Arrows indicate ^{32}P-labeled DNA and DNA/PNA target molecules.

The results presented in this article demonstrate that cationic submicron particles can be proposed for in vitro delivery to target cells of PNA/DNA hybrids. The simplicity and the versatility of the cationic submicron particle technology have made these reagents useful as nonviral gene delivery systems for human gene therapy.[9-11] Further studies are in progress to assess the ex vivo effectiveness of submicron particles for administration of biologically active PNA/DNA hybrids.

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